Inhibitory Potential of Four-Carbon Dicarboxylic Acids on *Clostridium botulinum* Spores in an Uncured Turkey Product

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ABSTRACT

Organic acids offer promising options for the food industry in its attempt to ensure product safety and to meet consumer demand for minimally processed foods. In this study, four-carbon dicarboxylic acids were individually screened for their inhibitory potential against proteolytic *Clostridium botulinum* spores. Ground turkey breast meat was formulated with 1.4% sodium chloride (NaCl), 0.3% sodium pyrophosphate, 2% organic acid, 8% water and 500 spores/g of a six-strain mixture of proteolytic *C. botulinum*. Samples were adjusted to pH 6. Ten g of product in vacuum packages were heated in 75°C water for 20 min, cooled and incubated for 0 to 25 days at 28°C. Botulinal neurotoxin was detected at two days in control samples (0% acid) and at five days in 2% malic acid (0.13 M), aspartic (0.13 M), tartaric (0.12 M), succinic (0.15 M), fumaric (0.15 M) samples. Toxin was undetected at 25 days in samples treated with maleic acid (0.15 M). Maleic acid reduced total aerobic bacteria and lactic acid organisms in temperature-abused product, compared to controls. Further systematic investigation of these and related compounds with prior approval for food-use may demonstrate previously unrecognized antibacterial potential.

Key Words: Four carbon dicarboxylic acids, *Clostridium botulinum*, antibotulinal activity

Previous research has shown that certain organic acids and their salts that are derived from plants, animals and microorganisms exhibit activity against the sporeforming anaerobic pathogen *C. botulinum*. These include lactate, acetate, propionate and citrate (*15,16*). Concern about hazards associated with *C. botulinum* stem from the widespread distribution in soils and sediments of the thermotolerant spores and their growth potential in low acid temperature-abused refrigerated foods. Organic acids offer promising options for the food industry in its attempt to ensure product safety and to meet consumer demand for minimally processed foods.

Uncured turkey breast is a popular deli product that currently lacks natural or formulated barriers to *C. botulinum* growth and toxin production, other than refrigeration. Although proteolytic strains will not germinate below 10°C, temperature abuse during storage and transportation can occur, thus creating a potential for growth and toxin production. Moreover, nonproteolytic strains are psychrotrophic and can grow at 3.3°C (*18*). Concerns about the safety of such products also arise because competitive bacteria are eliminated or sharply reduced by the mild heat treatment, and because of the anaerobic state created by vacuum-packaging (*17*). Thus, additional barriers are required to ensure safety of this product.

Huhtanen et al. (*12*) screened a host of potential nitrite substitutes and demonstrated that the synthetic compound maleic acid, a four-carbon alkenoic dicarboxylic acid, was an active inhibitor against *C. botulinum* spores in comminuted bacon. This observation prompted us to test related naturally-occurring four-carbon dicarboxylic acids for their inhibitory potential against *C. botulinum* spores in a product that was designed to model a refrigerated uncured turkey breast.

MATERIALS AND METHODS

Spore cultures

Six proteolytic strains of *C. botulinum*, three type A (62A, 69, 33) and three type B (169, FDA 999, C11) were heat shocked (80°C for 10 min), then anaerobically sporulated inside of a flexible anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI) in botulinal assay medium broth (*9*) at 35°C. After approximately two weeks, spores were harvested by centrifugation, washed with sterile distilled water and enumerated by plating and incubating anaerobically at 35°C for 48 h on botulinal assay medium agar plates. Each strain was tested for homogeneity and diagnostic biochemical markers, as described previously (*16*). Equal numbers of the six strains were pooled for a final concentration of 1.0×10^7 spores/ml and stored at 4°C in sterile distilled water. For each experiment, the spore mixture was heat shocked at 80°C for 10 min and enumerated as described above.

Product formulation

Turkey breast meat was obtained from a local distributor, hand deboned and double ground with 3/8" then 3/16" grinder plates using a laboratory grinder (Hobart Corp., Troy, OH). Eight 500 g batches were prepared and each received 1.4% NaCl (7 g, wt/wt) and 0.3% sodium pyrophosphate (1.5 g, wt/wt; Fisher Scientific Company, Fair Lawn, NJ). In addition, 10 g (2%) of

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one of the following acids in 40 ml (8%) sterile double distilled, deionized water were combined (10% total), adjusted to pH 6 with sodium hydroxide (NaOH), and added to six of the batches: L-aspartic (Sigma Chemical Co., St. Louis, MO), fumaric (J. T. Baker Chemical Co., Phillipsburg, NJ), maleic (Sigma), DL-malic (Aldrich Chemical Co., Milwaukee, WI), succinic (J. T. Baker) and L-tartaric (Aldrich). The molar concentrations and proton dissociation characteristics of these acids are shown in Table 1. As needed, the pH levels of the turkey formulations were adjusted using 1 M HCl or NaOH to a target level of pH 6, with a pH meter equipped with a combination electrode (model PHM82, Radiometer A/S, Copenhagen). Food mixtures were blended with a Hobart model N-50 mixer (Hobart) for 1 min at the slowest speed. One of the two remaining batches was used as a positive control (inoculated with spores) and the other as a negative control (uninoculated).

TABLE 1. *Physical and chemical properties of four-carbon dicarboxylic acids used in uncured turkey samples at initial pH = 6.*

Acid	Formula weight	Experimental concentration (M)	pKa_1	pKa_2
Succinic	118.09	0.15	4.18	5.55
Aspartic	133.10	0.13	1.88	3.65
Malic	134.09	0.13	3.3	5.05
Tartaric	150.09	0.12	2.93	4.23
Fumaric	116.07	0.15	3.03	4.38
Maleic	116.07	0.15	1.94	6.23

Turkey product inoculation and incubation

Each formulation was placed into heat sealable plastic bags, weighed and a target level of 500 spores/g was inoculated into all but the negative control by adding a diluted suspension (ca. 5 ml) of the spore mixture. Bags were heat sealed and mixed well by hand kneading to ensure equal spore distribution. After mixing, 10 ± 0.1 g samples were weighed into filter Stomacher bags (Tekmar, Cincinnati, OH). Stomacher bags were folded and placed into high oxygen barrier bags (O_2 permeation = 3.5 cc/100 in^2/24 h at 24°C and 75% relative humidity), vacuum sealed to -950 Mbar (Multivac Model GK110, Smith Equipment Co., Clifton, NJ), heated in 75°C water for 20 min, cooled in crushed ice and incubated at 28°C for 0 to 25 days. At the end of each incubation period, samples were frozen at -18°C until tested for neurotoxin. Three replicate samples per treatment were tested at each designated sampling time. Duplicate experiments were performed.

Product endpoint pH

Twenty ml of sterile distilled water was added to the turkey formulations in each Stomacher bag and the contents were then macerated with a Model 400 Stomacher (A. J. Seward, London) for two min. Filtrates were transferred into sterile tubes and pH levels were measured using a combination electrode (Radiometer A/S).

Bacterial enumeration

A portion of each sample filtrate was transferred into 15 ml sterile test tubes. Each filtrate was serially diluted in 0.1% peptone water and plated in duplicate on both de Man Rogasa Sharpe (MRS) agar plates (Difco Laboratories, Detroit, MI) and Nutrient agar plates (Difco) using a spiral plater (Spiral Plater Model D, Spiral System, Cincinnati, OH). De Man Rogasa Sharpe agar plates were incubated at 35°C inside of a flexible anaerobic chamber (Coy), and Nutrient agar plates were incubated aerobically at 37°C. Plates were counted manually using a colony counter pen (Manostat, New York, NY) and a template (Model MV, Spiral System). The limit of detection was 21 CFU/g.

Neurotoxin bioassay

After pH measurements were performed the filtrates were centrifuged at 1500 × g for 5 min in a model GLC-1 clinical centrifuge (Sorvall, Newtown, CT). Each supernatant fluid was tested for botulinum neurotoxin by intraperitoneal injection of 0.5 ml into each of two 15 to 20 *g* albino mice (West Jersey Biological Co., Wenonah, NJ). Mice were acclimated for 24 h prior to challenge, then observed for 48 h post-injection for typical botulinal symptoms according to customary practices (*7*). Selected samples were neutralized following procedures outlined by the Food and Drug Administration (*7*) with types A, B and polyvalent antitoxins (Centers for Disease Control and Prevention, Atlanta, GA) to confirm clinical observations.

Sensory evaluation

Samples were evaluated by at least one experienced investigator for evidence of off-odors (pungent, putrefied) and textural changes (mushiness, exudate, friability). All investigators were immunized against botulinum neurotoxin.

RESULTS

pH

Although the pH level of the raw formulation was adjusted to 6.0, a uniform rise was observed after the 20 min 75°C heat treatment, as shown in Fig. 1. Post-heating pH values were 6.37 for the uninoculated control and 6.44 for the positive control. For acid-treated samples post-heating pH values were 6.35, 6.41, 6.39, 6.25, 6.35 and 6.31 for aspartic, fumaric, malic, succinic, tartaric and maleic acids, respectively. The incubation period was concluded when two sequential samples tested positive for neurotoxin. The sample pH values at the time when toxicity was first detected were 6.46, 6.45, 6.44, 6.24, 6.36, 6.62 and 6.28 for aspartic, fumaric, maleic succinic, tartaric, uninoculated control and spore-containing control, respectively. At 25 days maleic acid samples remained toxin-free and had an average pH value of 6.44.

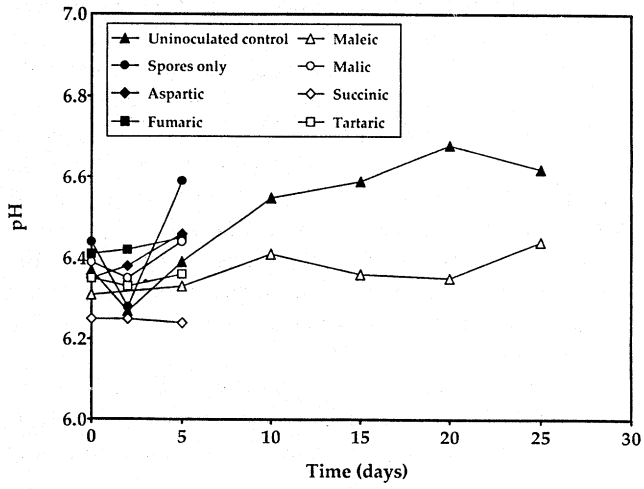

Figure 1. *pH changes in vacuum-packaged uncured turkey samples stored at 28° containing 2% four-carbon dicarboxylic acids and inoculated with 500 spores/g* C. botulinum.

Total aerobic growth

Results from aerobic growth at 37°C on Nutrient agar are shown in Fig. 2. After heating for 20 min at 75°C initial microbiota levels on organic acid-treated samples were at the minimum level of detection (<21 CFU/g). By five days total aerobic bacterial density was 4.6, 4.4, 1.8, 4.2, 2.9 and 2.6 log CFU/g for aspartic, fumaric, malic, succinic, tartaric and maleic acids, respectively. Maleic acid samples averaged 2.5 log CFU/g at the end of the 25 days incubation period at 37°C. Negative control samples had initial microbiota levels of <21 CFU/g at day 0 and 4.7 at 5 days, while positive control samples had total bacterial densities of 1.3 log CFU/g and 4.2 log CFU/g for 0- and 5-day samples, respectively. There were 5.8 log CFU/g in the 25-day negative control samples.

Lactic acid bacteria

Lactic acid bacteria were determined by enumeration on MRS agar, after anaerobic incubation at 35°C (Fig. 3). After heating for 20 min at 75°C, initial lactic acid bacteria

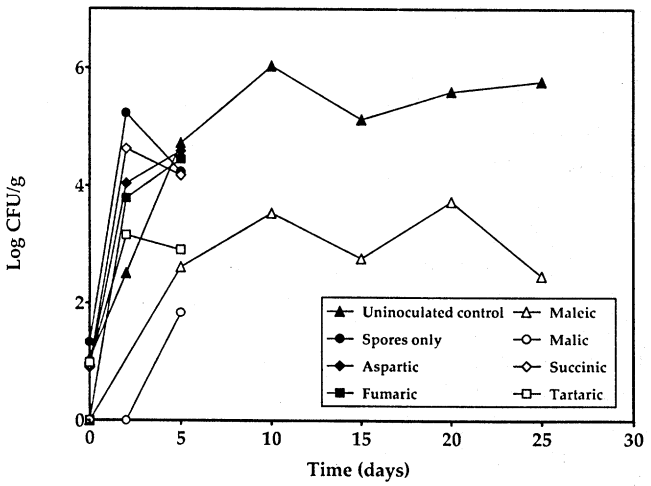

Figure 2. *Total aerobic plate counts in vacuum-packaged uncured turkey samples stored at 28° containing 2% four-carbon dicarboxylic acids and inoculated with 500 spores/g* C. botulinum.

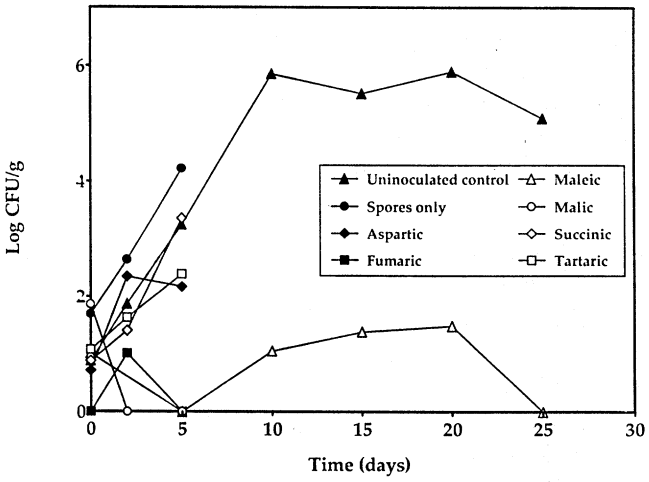

Figure 3. *Lactic acid bacteria in vacuum-packaged uncured turkey samples stored at 28° containing 2% four-carbon dicarboxylic acids and inoculated with 500 spores/g* C. botulinum.

levels on organic acid-treated samples were <21 CFU/g to 1.9 log CFU/g. Initial control samples contained <21 CFU/g and 1.7 log CFU/g, for negative and positive control samples, respectively. By five days, lactic acid bacteria levels were 2.2, 3.4 and 2.4 log CFU/g for aspartic, succinic and tartaric acid, respectively, and <21 CFU/g for fumaric, malic and maleic acids. Control five-day samples contained 3.3 and 4.2 log CFU/g for negative and positive control samples, respectively. Maleic acid samples averaged <21 CFU/g at the end of the 25-day incubation period at 35°C. There were 5.1 log CFU/g in the 25-day negative control samples.

Neurotoxin development

Botulinal neurotoxin data are shown in Table 2. Neurotoxin was detected at two days in 0% acid samples and at five days with 2% malic (0.13 M), aspartic (0.13 M) succinic (0.15 M), fumaric (0.15 M) and tartaric (0.12 M) acid. Toxin was undetectable at 25 days in samples treated with maleic acid (0.15 M). A few of the negative controls exhibited neurotoxin during the 25-day experimental period. Upon rechallenge with 1:3,000 dilutions, these samples induced in mice typical clinical botulinal symptoms of rough coats, progressing to pinched abdomens, then bellows breathing, within 6 h post-injection. Neurotoxin was confirmed in presumptive-positive food extracts by survival after rechallenge with antiserum neutralized samples.

TABLE 2. *Toxin detection times for vacuum-packaged turkey samples at 28°C containing 2% four-carbon dicarboxylic acids and inoculated with* C. botulinum spores[a].

Treatment (Concentration, M)	Time to toxin detection (Days at 28°C)						
	0	2	5	10	15	20	25
No acid	0/6[b]	5/6	6/6				
Aspartic (0.13)	0/6	0/6	6/6	3/3			
Fumaric (0.15)	0/6	0/6	6/6	3/3			
Maleic (0.15)	0/6	0/6	0/6	0/6	0/6	0/6	0/6
Malic (0.13)	0/6	0/6	6/6	3/3			
Succinic (0.15)	0/6	0/6	6/6	3/3			
Tartaric (0.12)	0/6	0/6	6/6	3/3			

[a] 500 spores/g added of a mixture of six proteolytic A and B strains.

[b] Number of positive samples/total packages.

Organoleptic evaluation

Positive control samples exhibited textural friability and a pungent odor at 2 days, while all acid-treated samples except maleic were unacceptable for consumption at five days. Maleic acid remained acceptable by smell and texture during the 25-day testing period.

DISCUSSION

Four-carbon dicarboxylic acids were evaluated for antibotulinal activity in a temperature-abused uncured turkey product, formulated to simulate a deli-type, cook-in-the-bag turkey roll. None of the naturally-occurring acids in

this class were effective, while 2% maleic acid inhibited *C. botulinum* neurotoxin production through 25 days of 28°C temperature abuse. The observation that a few of the uninoculated control samples contained botulinal neurotoxin after incubation at 28°C is consistent with observations by other investigators who reported that *C. botulinum* types A and B spores are found in terrestrial muscle foods at <0.1 to 7 spores per kg (*5*). The observation underscores the need for barriers to microbial growth in products at-risk for temperature abuse.

In the present study, total aerobic and lactic acid bacterial levels were inhibited by maleic acid, with respect to controls. These results are in agreement with de Wit and Rombouts (*4*), who indicated that addition of organic acids generally reduces spoilage organism levels, thus increasing overall product shelf-life, as well as enhancing microbiological safety. The marginally lower pH observed in maleic acid-treated samples most likely resulted from the inhibitory effect on the spoilage microbiota that would produce alkaline breakdown products, such as ammonia.

Previous research has demonstrated that monocarboxylic acid salts effectively delayed *C. botulinum* toxigenesis in a similar product (*15,16*). A structure-activity analysis showed that the antibotulinal efficacy of these compounds was directly related to higher pKa_1. Maas et al. (*15*) hypothesized that the mechanism of action of sodium lactate inhibition was either membrane transport inhibition of a critical substrate or feedback inhibition of the ATP-generating metabolic pathway. Miller et al. (*16*) confirmed the antibotulinal activity of lactate and demonstrated efficacy of acetate and propionate. These investigators also showed that the tricarboxylic acid salt, sodium citrate, was effective as a *C. botulinum* inhibitor in an uncured turkey product. In that study 0.2 M citrate delayed neurotoxin development until 18 days of temperature abuse at 28°C. Using a model system Graham and Lund (*10*) showed that citrate delayed *C. botulinum* neurotoxin development by chelation and subsequent mineral depravation.

Huhtanen et al. (*12*) screened short-chain alkynoic and alkenoic acids and esters for gas production inhibition by *C. botulinum* in temperature-abused cans of comminuted, nitrite-free bacon. Maleic acid was found to be effective, but the compound was rejected at that time for further study, because it was found that alkyl-esters were more efficacious against *C. botulinum*. Huhtanen (*11*) and Dymicky et al. (*6*) showed antibotulinal activity by fumarate and maleate esters, with C_8 to C_{15} derivatives exhibiting the greatest activity. Islam (*13*) showed that dimethyl fumarate inhibited mold growth on bread loaves during 475 days of storage.

The mechanism responsible for the antibacterial activity of maleic acid is unknown. An evaluation of the similarities and differences of the chemical properties of the compounds used in this study, however, may provide insights for further experimentation. None of the dicarboxylic acids studied are readily permeable to spores or vegetative cells at the experimental pH. For example, using the dissociation constants for maleate, given in Table 1, only 1.3×10^{-5} M of the 0.15 M total maleic acid would theoretically remain in the unionized form at pH 6. This suggests that the compound either does not passively diffuse to the cell interior, or the inhibitory site is at the surface. If the compound is internalized, it is likely that maleate enters the cell through an active or passive transport mechanism, although we have provided no data here to support or refute either alternative. Chemically, the stereoisomers maleic (cis) and fumaric (trans) acids possess high electron-withdrawing capacity because of the carboxyl groups in the conjugated system, thus making them both susceptible to attack by nucleophilic reagents (*14*). It is conceivable that if internalized either compound could bind and inactivate one or more critical cellular nucleophiles. The key factor differentiating maleate from all other compounds tested in this study, therefore, appears to be the stearic configuration of the carboxylate groups around the double bond.

Freese et al. (*8*) indicated that lipophilic acid antimicrobial agents uncouple both substrate transport and oxidative phosphorylation from the electron transport system. The exclusive use by *C. botulinum* of anaerobic respiration suggests that substrate transport inhibition would be a logical mechanism to test experimentally. In addition, Booth (*1*) indicated that organic acids may inhibit growth by acidifying the cell, consuming energy to maintain pH balance, or affecting enzymatic reaction rates. Other potential mechanisms for inhibition by maleate include inhibition of amino acid degradation, a primary energy source for proteolytic *C. botulinum* strains.

Regarding the toxicity of maleic acid, it has a mouse oral LD_{50} of 2,400 mg/kg and has antineoplastic activity in animal models (*2*). Chronic feeding to rats exhibited toxic effects at a concentration as low as 0.5% of diet, although the pathology was non-specific (*3*). Pharmacokinetic tests have demonstrated that the kidneys are the principal target organ in animal models (*3*).

Maleic acid is not a GRAS substance, and, unlike the five other acids tested in this study, it does not occur in nature. Rather, maleic acid is synthesized from maleic anhydride, as is synthetically-derived fumaric acid. It has a number of manufacturing applications (*14*), including the over-the-counter antihistamine chlorpheniramine, which is prepared as the maleate salt. Studies are continuing to develop a better understanding of the mechanism of inhibition of *C. botulinum* by maleic acid. Furthermore, continued systematic investigation of related compounds with prior approval for food use may demonstrate previously unrecognized antibacterial potential.

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